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Retinol and retinal metabolism

Relationship to the state of differentiation of cultured human keratinocytes

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Cultured keratinocytes offer an attractive model for studying the metabolism of retinol in relation to cell differentiation, since the extent of keratinocyte differentiation can be modulated experimentally. The metabolism of retinol and retinal was studied in cytosol fractions prepared from two distinct keratinocyte populations, differentiating and non-differentiated. The enzymic activities were analysed using physiological concentrations of [^{3}H]retinol and [^{3}H]retinal in the presence of cofactors. The products formed were quantified by h.p.l.c. In the population of differentiating keratinocytes, the formation of retinoic acid from retinol occurred at a rate of 4.49 ± 0.17 pmol/h per mg of protein, but no such conversion was observed in the population of non-differentiated cells. However, when retinal was used as substrate, retinoic acid was formed in both cell populations, at rates of 14.4 pmol/h per mg of protein in non-differentiated and 51.6 pmol/h per mg of protein in differentiating keratinocytes. Using PAGE/radiobinding assay, we demonstrated that retinoic acid formed from retinol was bound in differentiating keratinocytes to endogenous cellular retinoic acid-binding protein (CRABP). Furthermore, retinal was reduced to retinol in the presence of NADH in both differentiating and non-differentiated keratinocytes at a similar rate (8 pmol/h per mg of protein). Although retinal could not be detected under physiological conditions, it was found in significant amounts at pH 8.5–9, which is optimal for enzymic activity. This indicates that in keratinocytes retinal is an intermediate metabolite in retinoic acid formation from retinol. The enzymes catalysing the conversion of retinol into retinoic acid were found to differ from other alcohol and aldehyde dehydrogenases, since the formation of retinoic acid was not significantly affected by specific inhibitors of alcohol metabolism, such as 4-methylpyrazole and disulfiram. Moreover, the cytosol of non-differentiated keratinocytes did not generate retinoic acid from retinol despite showing alcohol dehydrogenase activity. The results suggest that: (1) retinol metabolism in human keratinocytes is different from that of other alcohols, (2) retinal is an intermediate metabolite in the conversion of retinol into retinoic acid, and (3) differentiating keratinocytes rich in CRABP are probably target cells for retinoic acid action.

INTRODUCTION

It is commonly accepted that retinol is essential for vision, reproduction, growth and cellular differentiation [1]. In contrast, retinoic acid has been shown to be involved only in the regulation of cell differentiation and growth (reviewed in [2]). This is consistent with observations that vitamin A-deficient animals fed with retinoic acid can grow normally but become sterile [3,4] and blind [5]. It has been assumed that retinoic acid is a physiologically important metabolite of retinol, representing an active form of vitamin A in some specific functions, and that the conversion of retinol into retinoic acid may be partially responsible for the activity that had been earlier attributed to retinol [6]. Since the concentration of retinoic acid in human serum is very low [7], approx. 150 times lower than that of retinol, and since retinoic acid is rapidly eliminated [8], it is suspected that retinoic acid is formed from retinol near to the site of its action, probably within the target cells. The presence of retinol esters stored in human epidermal cells [9] supports this hypothesis.

It has been assumed that retinoic acid is formed enzymically from retinol by a two-step oxidation process in which retinal is an intermediate metabolite [10]. However, retinal has never been detected in various tissues or in various types of cultured cells. Studies on the oxidation of retinol to retinoic acid in rat liver and kidney [11] and mouse epidermis [12] showed that the presence of NAD^+ is required for these reactions. Since many tissues, including liver and skin, contain high levels of alcohol and aldehyde dehydrogenases [13,14], it remains an open question whether the dehydrogenases involved in retinoid metabolism are related to those involved in alcohol metabolism.

At present, insufficient information is available on the metabolism of retinoids in human epidermal cells and on its potential changes in relation to the differentiation of these cells. Recently we have found [15] that the level of cellular retinoic acid-binding protein (CRABP) was elevated in a population of differentiating cultured human keratinocytes, whereas keratinocytes that were maintained in a non-differentiated state contained much less CRABP. On the basis of these findings we speculated that the conversion of retinol into retinoic acid may also be affected by the differentiation state of the cells. Therefore the present investigation was undertaken to study the metabolism of retinol at physiological concentrations and of retinal in two different populations of cultured keratinocytes: differentiating and non-differentiated. The results show that metabolism of retinol and retinal differs markedly in these two populations. The enzyme systems involved were found to differ from those involved in general alcohol metabolism. Furthermore, only in the population of differentiating keratinocytes was formation of retinoic acid

Abbreviations used *m*-AMSA, 4′-(9-acridinylamino)methanesulphon-*m*-anisidine; CRABP, cellular retinoic acid-binding protein: CRBP, cellular retinol-binding protein; ADH, alcohol dehydrogenase.

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from retinol observed. During this conversion, retinal was found to be the intermediate metabolite.

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EXPERIMENTAL

Materials

Retinol and retinoic acid were purchased from Sigma. 13-*cis*-Retinoic acid and all-*trans*-4-oxo-retinoic acid were gifts from Dr. M. Klaus, Hoffmann-La Roche (Basel, Switzerland). [11,12-^{3}H]All-*trans*-retinol (50 Ci/mmol) was obtained from Dupont–NEN, Bad Homburg, Germany. [^{3}H]Retinal was prepared from [^{3}H]retinol by MnO_2 oxidation in hexane as described [16] and was purified further on a reversed-phase h.p.l.c. column, as described below. 4-Methylpyrazole and disulfiram were purchased from Aldrich. *m*-AMSA was a gift from Dr. J.-L. Napoli, State University of New York, Buffalo, NY, U.S.A. All operations involving the retinoids were performed under yellow light. The purity of the labelled and unlabelled retinoids was checked by h.p.l.c. analysis and consistently exceeded 95%. All alcoholic stock solutions of retinoids contained butylated hydroxytoluene (50 μg/ml) as antioxidant.

Cell culture

Keratinocytes originating from juvenile foreskin were cultured using the Rheinwald–Green feeder technique [17]. The medium used was a mixture of Dulbecco–Vogt and Ham's F12 media (3:1, v/v) supplemented with 5% fetal calf serum, 1 μM-isoprenaline [18], 1.1 mM-cortisol and 10 ng of epidermal growth factor/ml [19]. When the switch from normal Ca^{2+} (1.6 mM) to low Ca^{2+} (0.06 mM) was performed, the keratinocytes were first grown to confluency at normal Ca^{2+} concentrations and subsequently incubated for 2–3 days in medium containing a low Ca^{2+} concentration [20]. During this period, the more highly differentiated cells detached from the culture and were shed into the culture medium. They were collected by centrifugation of the medium and washed three times with ice-cold calcium- and magnesium-free phosphate-buffered saline (PBS; 5 mM-Na_2HPO_4/1.54 mM-KH_2PO_4/130 mM-NaCl, pH 7.2). The less-highly differentiated cells that remained attached to the culture dish were washed three times with PBS before being scraped off with a rubber policeman. The cells were stored at −70 °C until use. To minimize problems of understanding the terms, 'non-differentiated' will be used for attached cells, whereas 'differentiating' will be used for shed keratinocytes.

Preparation of cytosol fraction

About 35 mg of lyophilized keratinocytes was first homogenized three times at full-speed for 30 s in 600 μl of extraction buffer (50 mM-Tris/HCl, 25 mM-NaCl, 2.5 mM-EDTA and 1 mM-dithiothreitol, pH 7.5) using a tissue homogenizer Polytron-PT7 (Kinematica, Lucerne, Switzerland) and subsequently centrifuged at 100000 *g* for 60 min at 4 °C. Protein content of the cytosol was determined by the method of Lowry *et al.* [21].

Retinoid assays

The following standard incubation conditions were used to assay retinoic acid synthesis. The required volumes of alcoholic solutions of retinoids (in glass tubes) were evaporated under N_2. Subsequently, 100 μl of cytosol fraction (0.15–0.25 mg of protein) containing 2 mM-NAD^+ was added and the mixture was incubated for 1 h at 37 °C in a shaking water bath. Concentrations of [^{3}H]retinol and [^{3}H]retinal were 600 nM. As described in the text, cofactors or tested compounds were also added to the incubation mixture. Each experiment was performed at least two or three times, and no substantial differences were observed between individual experiments.

Measurement of CRABP and cellular retinol-binding protein (CRBP) activities during retinol metabolism

To assay CRABP and CRBP contents, the cytosol fraction of differentiating keratinocytes after incubation with [^{3}H]retinoic acid and [^{3}H]retinol was submitted to PAGE [22]. The amount of retinoic acid formed was measured by assaying the radioactivity associated with CRABP after incubation with [^{3}H]retinol. The purity of [^{3}H]retinol was checked by h.p.l.c. and no labelled retinoic acid was detected.

PAGE (slab gels) was run under dim light at 13 °C as previously described [22,23]. After electrophoresis, the individual lanes were cut into 2 mm bands, and proteins were hydrolysed in 250 μl of Protosol (NEN). The protein-bound radioactivity was measured using Pico-Fluor 15 (Packard) as scintillation fluid. For assessment of non-specific binding, the samples were incubated in the presence of [^{3}H]retinoids and an excess (200-fold) of non-labelled retinoid.

Analyses of retinoid metabolites by h.p.l.c.

After the incubation of a portion (100 μl) of cytosolic fraction with [^{3}H]retinol or [^{3}H]retinal, the reaction was terminated by the addition of 10 μl of 0.1 M-HCl and 100 μl of ethanol containing butylated hydroxytoluene (50 μg/ml). Subsequently, 5 μl of a solution of internal standards (4-oxo-retinoic acid, all-*trans*-retinoic acid, 13-*cis*-retinoic acid, retinol and retinal) was added, and the retinoids were extracted with 3 × 1 ml of hexane. The organic phase was collected and evaporated to dryness under a stream of N_2, then the residue was dissolved in 50 μl of acetonitrile and applied to a reversed-phase h.p.l.c. column. H.p.l.c. was performed using Varian 5000 equipment with reversed-phase column of ODS-Ultrasyl (octadecylsilane bonded phase, 10 μm particles, 25 cm × 0.4 cm) (Beckman, Geneva, Switzerland). Isocratic elution was performed using a solvent system composed of (v/v) 71% acetonitrile, 23% ammonium acetate (50 mM, pH 7) and 6% tetrahydrofuran at a flow rate of 2.4 ml/min, as described previously [24]. Non-labelled retinoids were detected by u.v. absorbance at 340 nm. Fractions of 600 μl were collected and their radioactivity was determined by liquid scintillation counting after the addition of 4 ml of Pico-Fluor 15. Extraction efficiencies, based on the recoveries of the known amount of [^{3}H]retinol or [^{3}H]retinoic acid added to the heat-denatured cytosol, were higher than 90 and 80% respectively. In totality, 50% of retinoic acid was recovered.

Methylation of retinoids

Diazomethane was generated using a diazomethane-generation apparatus using instructions provided by the manufacturer (Aldrich, Steinheim, Germany). Methyl-all-*trans*-retinoic acid standard was prepared by reaction under N_2 of all-*trans*-retinoic acid dissolved in ethanol and an etheral solution of diazomethane. The radioactive material recovered from each sample after its incubation with [^{3}H]retinol and after the evaporation of the organic phase was dissolved in 500 μl of absolute ethanol containing 50 μg of butylated hydroxytoluene/ml. Methylation was performed by mixing this solution with 500 μl of ethereal diazomethane solution (0.17 mmol). Subsequently, the solvents were evaporated under N_2 and the residue was dissolved in 50 μl of acetonitrile, and h.p.l.c. analysis was performed.

Methyl-all-*trans*-retinoic acid was separated from other retinoids by modifying the elution solvent system to 73.6% aceto-

nitrile, 21% ammonium acetate (50 mM, pH 7) and 5.4% tetrahydrofuran. Methyl-all-*trans*-retinoic acid had a retention time of 15.4 min.

Alcohol dehydrogenase (ADH) assay

The activity of ADH was measured photometrically at 340 nm by monitoring the production of NADH at 25 °C, according to Bühler & von Wartburg [25] adapted for keratinocytes. The assay was performed in a total volume of 1 ml of 35 mM-sodium pyrophosphate buffer, pH 8.8, containing 1.25 mM-NAD^+ and a portion of cytosol fraction (250 μg of protein). The reaction was started by the addition of 15 mM-hexenol or -ethanol. Activities are expressed as nmol of NADH produced/h per mg of protein.

RESULTS

[^{3}H]Retinoic acid–CRABP levels as a measure of [^{3}H]retinol metabolism

For this purpose PAGE was used, since it permits simultaneous measurement of CRABP and CRBP levels in the same lane of the gel [22,23]. After incubation of cytosol from differentiating keratinocytes with [^{3}H]retinol, both radioactive CRBP (band no. 38) and CRABP (band no. 24) peaks were detected (Fig. 1*a*). Incubation in the presence of a 200-fold excess of nonlabelled retinoic acid diminished substantially the surface area of the radioactive CRABP peak, but not that of the CRBP peak. When the incubation was carried out in the presence of an excess of non-labelled retinol, both CRABP and CRBP radioactive peaks were almost completely abolished. Furthermore, with [^{3}H]retinoic acid, a [^{3}H]retinoic acid–CRABP peak (band no. 24) was observed. This peak was almost totally abolished when the incubation was carried out in the presence of a 200-fold excess of either non-labelled retinoic acid or non-labelled retinol (Fig. 1*b*).

The amount of the [^{3}H]retinoic acid–CRABP complex formed during the incubation of cytosol with [^{3}H]retinol increased with prolongation of the incubation time, reaching a maximum within about 2 h (Fig. 2). These results indicate that in this system, retinol is metabolized into a compound which is capable of binding to CRABP. When the experiments were carried out with cytosol obtained either from non-differentiated keratinocytes (that contain low levels of CRABP) or from cultured human and/or 3T3 fibroblasts (that contain high levels of CRABP [26]), no [^{3}H]retinoic acid–CRABP complex could be detected after incubation with [^{3}H]retinol.

Analysis of products of the enzymic oxidation of [^{3}H]retinol

After incubation of portions of cytosol fractions isolated from differentiating keratinocytes with [^{3}H]retinol, the retinoids were extracted and analysed by h.p.l.c. Fig. 3(*a*) shows the elution profiles of various internal standards measured at 340 nm. This choice of standards was made because these compounds are possible oxidation products of retinol. Fig. 3(*b*) shows a representative elution profile of the extracted radioactive material. Next to a large symmetrical peak co-eluting with the retinol standard, a second radioactive peak co-eluting with all-*trans*-retinoic acid was detected. The group of polar metabolites (eluting between fractions 5 and 18) and the small radioactive peak of retinal are not the products of the transformation of [^{3}H]retinol by enzymic oxidation, since they were also detected in the incubation mixtures containing cytosol which had been boiled for 10 min prior to the assay. The presence of radiolabelled retinoic acid was also confirmed by h.p.l.c. analysis on a reverse-phase column by its co-elution with anticipated methylation product all-*trans*-methylretinoate.

The radioactivity associated with CRABP detected in cytosol incubated with [^{3}H]retinol (see Fig. 1*a*) was also analysed by h.p.l.c. The bands corresponding to CRABP (fractions 23–27) were pooled, homogenized, centrifuged at 10000 *g* and the supernatant containing CRABP was extracted as described above. The h.p.l.c. analysis revealed the presence of one radioactive peak co-eluting with all-*trans*-retinoic acid (results not shown).

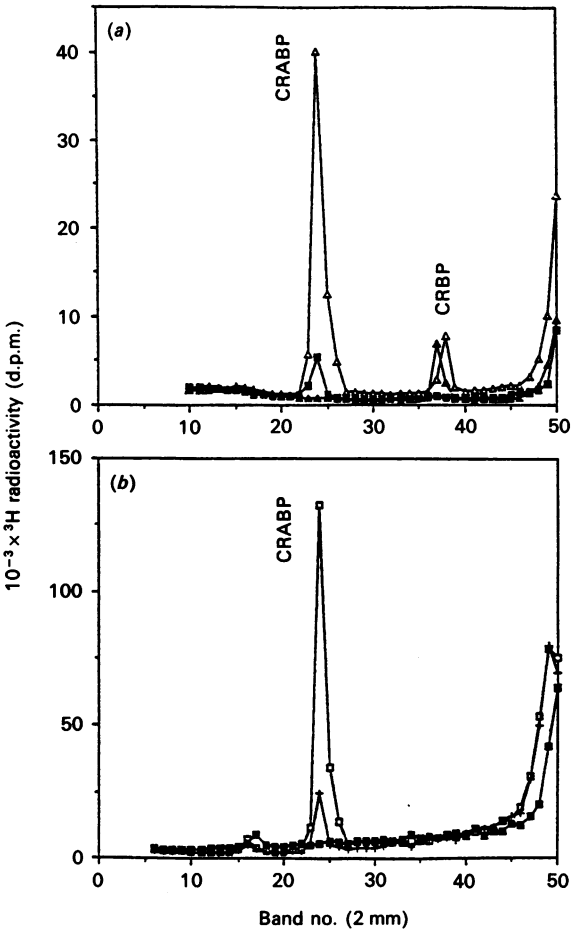

Fig. 1. PAGE analysis of retinoid-binding proteins in cytosolic fraction of human differentiating keratinocytes

(*a*) A portion of cytosol fraction, containing 250 μg of protein in 100 μl of buffer was incubated with 600 nM-[^{3}H]retinol for 1 h at 37 °C in the absence (△) or presence of a 200-fold molar excess of non-labelled retinoic acid (▲) or retinol (■). The migration positions of the first and second peaks correspond to CRABP (band 24) and CRBP (band 38) respectively, used as standards. The front migrates at band 50. (*b*) A portion of cytosol fraction containing 250 μg of protein in 100 μl of buffer was incubated with 600 nM-[^{3}H]retinoic acid for 1 h at 37 °C in the absence (□) or presence of a 200-fold molar excess of unlabelled retinoic acid (■) or retinol (+). The front migrates at band 49.

Characterization of the system transforming retinol into retinoic acid

In order to determine whether the conversion of retinol into retinoic acid is an enzyme-catalysed process, the cytosol fraction of differentiating keratinocytes was heated for 10 min at 95 °C. This treatment indeed prevents retinoic acid production. Experiments in which [^{3}H]retinoic acid formation was studied as a function of the [^{3}H]retinol concentration revealed that the system was saturated at 0.3 μM-[^{3}H]retinol (Fig. 4). The time course of [^{3}H]retinol oxidation showed that the decrease in [^{3}H]retinol

concentration correlated with the increase in [^{3}H]retinoic acid (Fig. 5). The rate of retinoic acid production remained constant up to 2 h and increased linearly with increasing protein content of the cytosolic fraction in the range 1–4.5 mg of protein (results not shown).

As shown in Table 1, the rate of conversion of retinol to retinoic acid in cytosol of differentiating keratinocytes was increased in the presence of 2 mM-NAD^+; however, in the cytosolic fraction of non-differentiated keratinocytes, no [^{3}H]retinoic acid was formed, even in the presence of NAD^+.

In order to characterize the process of retinoic acid formation in more detail, various compounds were added to the reaction mixture. The results of these experiments are given in Table 2. Two potent inhibitors of ethanol metabolism, 4-methylpyrazole (100 μM) [27] and disulfiram (100 μM) [28], were not effective. *m*-AMSA, an inhibitor of aldehyde oxidase and a potent inhibitor of retinol dehydrogenation in rat liver and kidney extracts [11], was only partially active. Pyridoxal 5-phosphate, an inhibitor of the membrane-associated retinol dehydrogenase activity in bovine rod outer segments [29], had no effect. In the presence of ethanol, an increase in [^{3}H]retinol oxidation occurred, probably as a result of increased solubility of [^{3}H]retinol in the medium.

Neither the differentiating nor the non-differentiated keratinocytes showed detectable ADH activity towards a short-chain alcohol such as ethanol. When an alcohol of a medium chain length (e.g. hexenol) was used as a substrate, the dehydrogenase activity in differentiating keratinocytes was 60.5±9 nmol/h per mg of protein (n = 7) and in non-differentiating cells it was

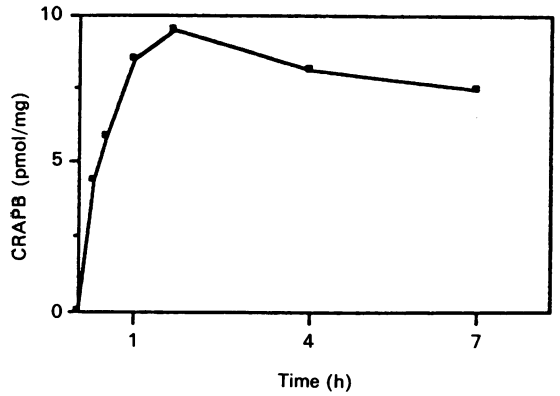

Fig. 2. Formation of CRABP–retinoic acid complex in differentiating keratinocytes

Cytosol fractions of differentiating keratinocytes containing 250 μg of protein were incubated for 0.5–7 h at 37 °C with 600 nM-[^{3}H]retinol. The amount of radioactive CRABP was measured by PAGE, as described in the Experimental section. The results are expressed as pmol of CRABP/mg of cytosol protein.

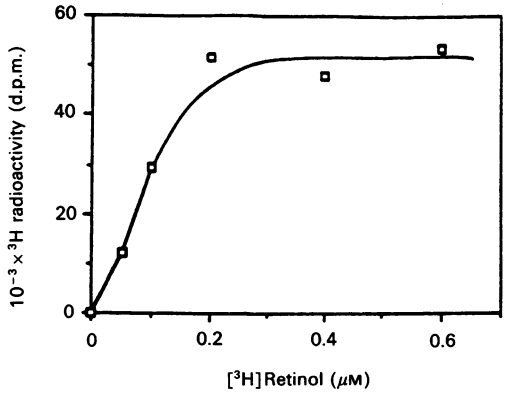

Fig. 4. Dependence of [^{3}H]retinoic acid formation on [^{3}H]retinol concentration

Samples (170 μg) of cytosolic proteins in 100 μl of buffer were incubated for 1 h at 37 °C with increasing concentrations of [^{3}H]retinol (0–0.6 μM). Samples were extracted and the amount of [^{3}H]retinoic acid was determined after h.p.l.c. analysis, as described in the Experimental section.

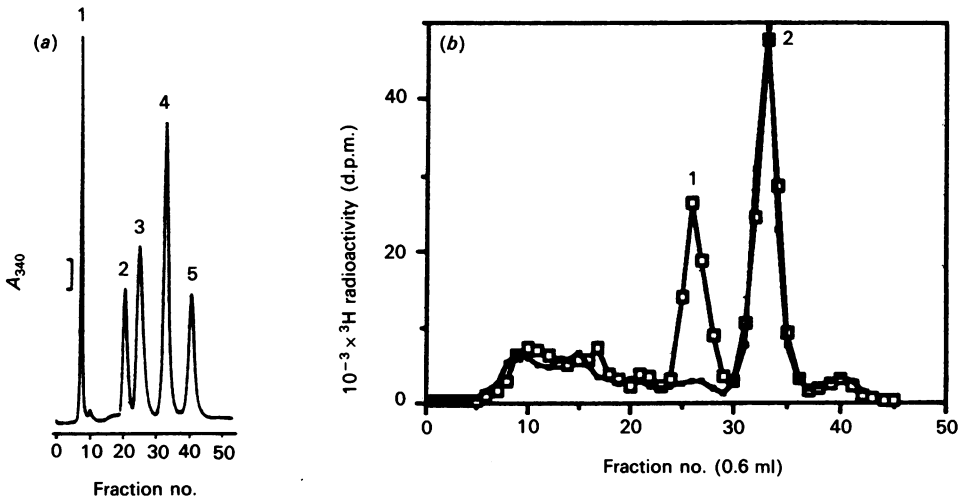

Fig 3. Metabolism of [^{3}H]retinol in differentiating keratinocytes

Portions of cytosol fraction (180 μg of protein in 100 μl of buffer) were incubated with 600 nM-[^{3}H]retinol for 1 h at 37 °C. Internal standards were added to the sample before the extraction of retinoids. The extracts were chromatographed on a ODS-Ultrasyl column, as described in the Experimental section. Absorbance was monitored at 340 nm; fractions (0.6 ml) were collected and the radioactivity was determined. (*a*) Profile of the internal standards 4-oxo-*trans*-retinoic acid (peak 1), 13-*cis*-retinoic acid (2), all-*trans*-retinoic acid (3), all-*trans*-retinol (4), all-*trans*-retinal (5); (*b*) radioactivity profile of retinoids in cytosol fraction, boiled (■) and not treated (□). Peak 1, retinoic acid; peak 2, retinol.

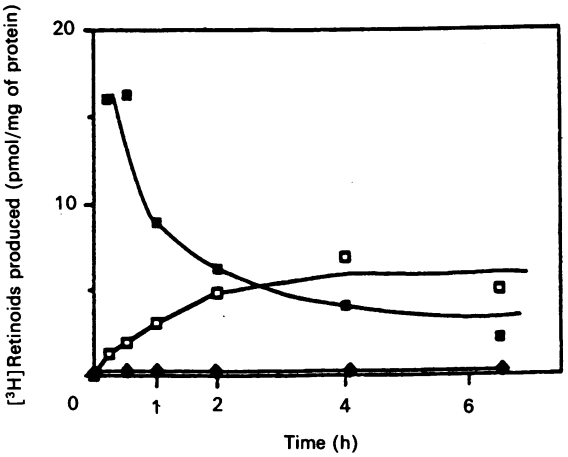

Fig. 5. Time course of [^{3}H]retinoic acid formation and of disappearance of [^{3}H]retinol in differentiating keratinocytes

Portions of cytosol (100 μl, containing 180 μg of protein) were incubated with 600 nM-[^{3}H]retinol for different time intervals ranging from 15 min to 6.5 h. After incubation, the samples were extracted and subjected to h.p.l.c. analysis for measurement of [^{3}H]retinol (■), [^{3}H]retinal (◆) and [^{3}H]retinoic acid (□) content.

Table 1. Rate of conversion of [^{3}H]retinol into [^{3}H]retinoic acid in differentiating and non-differentiated keratinocytes: effect of NAD^+

Results are means ± S.D. of three determinations

Keratinocytes	Addition...	[^{3}H]Retinoic acid formed (pmol/h per mg of protein) None	[^{3}H]Retinoic acid formed (pmol/h per mg of protein) NAD^+ (2 mM)
Differentiating		2.67 ± 0.25	4.49 ± 0.17
Non-differentiated		Trace	Trace

Table 2. Conversion of [^{3}H]retinol into [^{3}H]retinoic acid in the presence of various compounds

The data are expressed as the ratio of the rate of the conversion of [^{3}H]retinol into [^{3}H]retinoic acid in the presence and the absence of tested compound, multiplied by 100. −, inhibition; +, activation; none, no change. Values are means of two determinations.

Compound	Concentration (mM)	Change (%)
4-Methylpyrazole	0.1	−3.3
Disulfiram*	0.1	None
m-AMSA	0.1	−17.8
Ethanol	100.0	44.6
Pyridoxal 5′-phosphate	0.1	None

* Measured in the absence of dithiothreitol.

95.4 nmol/h per mg of protein ($n = 2$). These results show that in both cell populations the rate of hexenol oxidation is much higher than that of retinol oxidation and, furthermore, it is detectable in the non-differentiated keratinocytes in which no retinol oxidation occurred (Table 1).

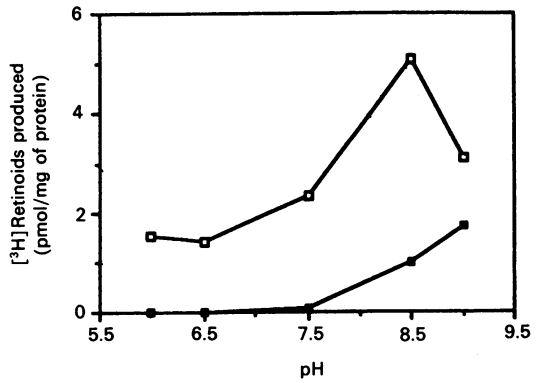

Fig. 6. Effect of pH on formation of [^{3}H]retinoic acid (□) and [^{3}H]retinal (■) from [^{3}H]retinol in differentiating keratinoyctes

The formation of [^{3}H]retinoic acid and [^{3}H]retinal from [^{3}H]retinol was measured using standard assay conditions (see the Experimental section), except that the pH in cytosol fractions was adjusted to the appropriate pH with 1 M-HCl or 1 M-NaOH.

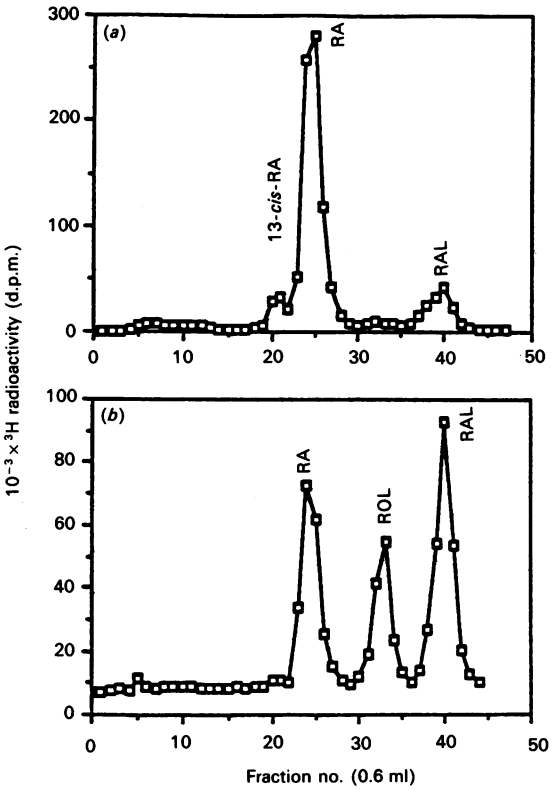

Fig. 7. Metabolism of [^{3}H]retinal in differentiating keratinocytes

Portions of cytosol fraction (100 μl, containing 150 mg of protein) were incubated with 600 nM-[^{3}H]retinal for 1 h at 37 °C in the presence of (*a*) 2 mM-NAD^+ or (*b*) 5 mM-NADH. Samples were extracted and subjected to h.p.l.c. analysis, as described in the Experimental section. The radioactive peaks co-eluted exactly with the corresponding internal standards. Abbreviations used: RA, retinoic acid; ROL, retinol; RAL, retinal.

Characterization of retinal metabolism

Under the experimental conditions used (i.e. at physiological pH), under which [^{3}H]retinoic acid was generated from [^{3}H]retinol in cytosolic extracts of differentiating keratinocytes, the levels of retinal were below the detection limit (Fig. 5). Since it has been proposed that retinal is an intermediate metabolite, we addressed

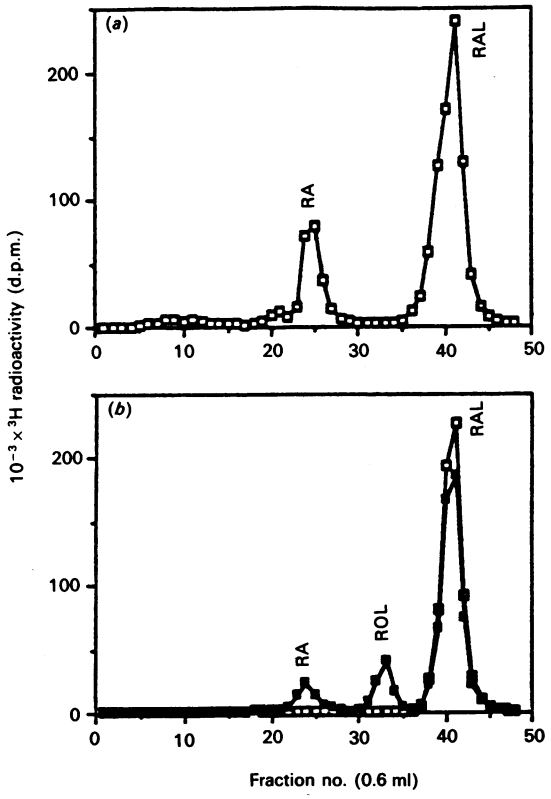

Fig. 8. Metabolism of [^{3}H]retinal in non-differentiated keratinocytes

Portions of cytosol fraction (100 μl, containing 130 μg of protein) were incubated with 600 nM-[^{3}H]retinal for 1 h at 37 °C, (*a*) in the presence of 2 mM-NAD$^+$, or (*b*) in the absence (□) or in the presence (■) of 5 mM-NADH. Samples were extracted and subjected to h.p.l.c. analysis. The radioactive peaks co-eluted exactly with the corresponding internal standards. Abbreviations used: RA, retinoic acid; RAL, retinal; ROL, retinol.

Table 3. Comparison of rates of conversion of [^{3}H]retinal into [^{3}H]retinol or [^{3}H]retinoic acid in relation to keratinocyte differentiation: effects of NAD$^+$ and NADH

Results are means of two determinations. The concentrations of NADH and NAD$^+$ used were 5 mM and 2 mM respectively.

		[^{3}H]Retinol formed (pmol/h per mg)		[^{3}H]Retinoic acid formed (pmol/h per mg)		
Keratinocytes	Addition...	None	NADH	None	NADH	NAD$^+$
Differentiating		3.6	8.2	9.5	13.1	51.6
Non-differentiated		0	8.0	0	4.5	14.4

the question of whether retinal is generated in the system studied. This appeared to be the case, since when the pH was raised to 9 in the incubation mixture, the formation of retinal became measurable (1.73 pmol/h per mg of protein). At this pH, a concomitant decrease in retinoic acid production was observed (Fig. 6). This indicates that retinal is an intermediate metabolite. Therefore the conversion of retinal into retinol or into retinoic acid was also studied. For this purpose, a portion of the cytosol fraction from differentiating keratinocytes was incubated with [^{3}H]retinal in the presence of cofactors. The products were analysed by h.p.l.c. as described above. As shown in Fig. 7(*a*), the

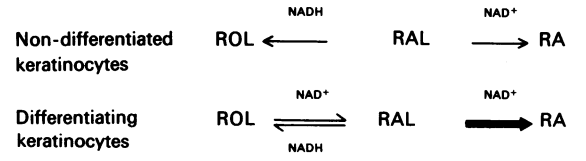

Fig. 9. Differences in retinoid metabolism pathways in human differentiating and non-differentiated cultured keratinocytes

This scheme was compiled from results presented in Tables 1 and 2. The arrows indicate the direction and the relative rate of the reactions in the presence of one of the different substrates and cofactors at saturation conditions. Abbreviations used: ROL, retinol; RAL, retinal; RA, retinoic acid.

formation of all-*trans*-retinoic acid was strongly stimulated in the presence of 2 mM-NAD$^+$. The small shoulder at fraction 21 corresponds to 13-*cis*-retinoic acid. Since the area under this peak never exceeded 10% of that of retinoic acid, the formation of 13-*cis*-retinoic acid can be considered to be a result of a non-specific isomerization. The radioactivity detected in fraction 40 corresponds to [^{3}H]retinal, used as substrate.

When 5 mM-NADH instead of NAD$^+$ was added to the incubation mixture, a peak of [^{3}H]retinol (fraction 33) was detected (Fig. 7*b*). During reduction of retinal into retinol, NADH is oxidized into NAD$^+$, which in its turn can be utilized for the oxidation of retinal into retinoic acid. This explains why, in the presence of NADH, [^{3}H]retinoic acid (fraction 24) was also detected.

As shown in Table 1, no transformation of retinol into retinoic acid occurred in non-differentiated keratinocytes. When the incubation was carried out with [^{3}H]retinal and in the absence of any cofactor, neither [^{3}H]retinoic acid nor [^{3}H]retinol was formed. However, when 5 mM-NADH was added, formation of [^{3}H]retinol (fraction 34) and to a lesser extent of [^{3}H]retinoic acid (fraction 25) was observed (Fig. 8*b*). The formation of retinoic acid might be explained by the presence of NAD$^+$ formed from NADH during retinal reduction. On the other hand, in the presence of 2 mM-NAD$^+$ the oxidation of [^{3}H]retinal into [^{3}H]-retinoic acid occurred (Fig. 8*a*). The results of these experiments are summarized in Table 3.

DISCUSSION

The results of the present study show that the commitment of normal human keratinocytes to terminal differentiation is associated with the expression of enzymic activity that mediates the transformation of retinol into retinoic acid. In contrast to this, a part of the enzymic activity, namely the retinol dehydrogenase activity, is lacking in cells that are not undergoing terminal differentiation (Fig. 9).

Human epidermis is a stratified keratinizing epithelium, the differentiation of which is dependent on the supply of natural retinoids [30]. Although retinoic acid is thought to be involved in this process, no information has been available as to whether it is delivered to the cells from the blood or whether it is formed by the keratinocytes themselves from retinol stored in these cells. Our findings show that cultured normal human keratinocytes are able to transform retinol into retinoic acid. This suggests that human epidermis itself can cover its needs for retinoic acid via an enzymic conversion of retinol. The finding that only keratinocytes that have been already committed to the terminal differentiation were found to fully express the enzymic system catalysing the transformation of retinol into retinoic acid supports the hypothesis that differentiating keratinocytes are predominantly the target cells for retinoic acid [31,32]. Further studies are needed for

exploring the possibility that the enzymic conversion of retinol into retinoic acid may represent a significant regulatory step in the differentiation of the epidermis. It is also unclear why high CRABP levels were only detected in the population of cells that are able to convert retinol into retinoic acid [15], with low levels in non-differentiated keratinocytes that are not able to transform retinol into retinoic acid. In the present study we demonstrated that retinoic acid generated from retinol was bound to CRABP already present in the cells. When we assume that CRABP functions as a regulatory element [33,34] which transports retinoic acid from the cytosol to the nucleus, we can suggest that, at least in cultured keratinocytes, CRABP may be an important physiological intermediate between the enzymic generation of retinoic acid and its binding to nuclear receptors [35].

The enzymic system that catalyses the conversion of retinol into retinoic acid in normal cultured human keratinocytes differs in several aspects from that described previously for other systems [12,29]: (1) its rate is low, (2) it is independent of ADH and (3) the conversion of retinol into retinal appears to be a rate-limiting step. These are discussed further below.

(1) The development and use of very sensitive techniques that allow the detection of retinol metabolites at femtomolar levels made it possible to measure the rate of retinoic acid formation in human keratinocytes, which was about 1000 times lower than that found in kidney pig cell lines [36] and rat tissues [11,12]. These techniques also made possible the use of physiological levels of retinol rather than the high concentrations that have been commonly used in previous studies by other authors. Due to this, the rate of retinol conversion measured here is probably close to that occurring *in vivo*. In fact, preliminary studies [37] have shown that the rate of retinol conversion in human epidermis is even lower than that in cultured keratinocytes.

(2) The enzymic conversion of retinol into retinoic acid in differentiating normal cultured human keratinocytes is not catalysed by ethanol dehydrogenase. Since we have used low levels of retinol, it is highly improbable that a non-specific ADH, that might be involved when high concentrations of retinol (1 mM) are used as a substrate [12], would interfere. Furthermore, specific inhibitors or competitors of ADH, such as ethanol, did not significantly inhibit the conversion of retinol into retinoic acid. Finally, further evidence that the involvement of ADH in retinol oxidation is improbable is the finding that ADH activities are similar in both differentiating and non-differentiated keratinocytes, whereas the transformation of retinol to retinoic acid was observed only in the population of differentiating keratinocytes. These data are in agreement with reports on other tissues in which retinol dehydrogenase has also been found to be different from ethanol dehydrogenase [36,38,39].

(3) Retinal plays the central role in the metabolism of natural retinoids. During the oxidation of retinol at physiological pH, all-*trans*-retinoic acid is the most prominent product, whereas retinal is found only in trace amounts. However, when the pH is raised to 9, retinal can be detected in significant amounts. This could explain the difficulties in detecting retinal in tissues [40,41]. The optimal pH for retinoic acid production was found to be 8.5, which is in agreement with the results of Napoli & Race [11]. In order to gain more information on processes involved in retinoic acid formation, the metabolism of retinal was studied in more detail. As shown in Table 3, both differentiating and non-differentiated keratinocytes have in their cytoplasm enzyme activities capable of both oxidizing and reducing retinal. Whether these activities are due to one or two enzymes remains to be established. These results are consistent with the observations on the developing chick limb bud *in vivo* [42]. Although non-differentiated keratinocytes are able to reduce retinal into retinol only in the presence of NADH and to oxidize retinal into retinoic acid only in the presence of NAD^+, the presence of these cofactors is not necessary for transformation of retinal in differentiating keratinocytes. This suggests that sufficient quantities of NADH and NAD^+ are present endogenously in those cells. The rate of conversion of retinal into retinol in the presence of NADH is similar in both differentiating and non-differentiated keratinocytes, whereas the rate of conversion of retinal into retinoic acid in the presence of NAD^+ is 3.5 times higher in differentiating keratinocytes. The finding that the rate of retinoic acid formation from retinal is higher than that from retinol suggests that the rate of formation of retinal, the first oxidation product of retinol, is the rate-limiting step of this process.

It should be noted that also another pathway exists for retinoic acid synthesis that does not require retinol as a substrate [41]. One of the possible candidates is β-carotene, since its cleavage involves [43] retinal as an intermediate metabolite. Indeed, β-carotene that has been found to be present in epidermal cells [9] and in serum (culture medium contains 10% fetal calf serum) may serve as a precursor of retinal. These findings suggest that retinal could play a central role in retinoid target tissues, and further studies are needed for the evaluation of the physiological role of retinal.

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